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The Hypervariable Immunodominant $NP_{418-426}$ Epitope from the Influenza A Virus Nucleoprotein Is Recognized by Cytotoxic T Lymphocytes with High Functional Avidity

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Recently it was shown that influenza A viruses can accumulate mutations in epitopes associated with escape from recognition by human virus-specific cytotoxic T lymphocytes (CTL). It is unclear what drives diversification of CTL epitopes and why certain epitopes are variable and others remain conserved. It has been shown that simian immunodeficiency virus-specific CTL that recognize their epitope with high functional avidity eliminate virus-infected cells efficiently and drive diversification of CTL epitopes. T-cell functional avidity is defined by the density of major histocompatibility complex class I peptide complexes required to activate specific CTL. We hypothesized that functional avidity of CTL contributes to epitope diversification and escape from CTL also for influenza viruses. To test this hypothesis, the functional avidity of polyclonal CTL populations specific for nine individual epitopes was determined. To this end, peripheral blood mononuclear cells from HLA-A- and -B-genotyped individuals were stimulated in vitro with influenza virus-infected cells to allow expansion of virus-specific CTL, which were used to determine the functional avidity of CTL specific for nine individual epitopes in enzyme-linked immunospot assays. We found that the functional avidity for the respective epitopes varied widely. Furthermore, the functional avidity of CTL specific for the hypervariable $NP_{418-426}$ epitope was significantly higher than that of CTL recognizing other epitopes ($P < 0.01$). It was speculated that the high functional avidity of $NP_{418-426}$-specific CTL was responsible for the diversification of this influenza A virus CTL epitope.

$CD8^+$ cytotoxic T lymphocytes (CTL) play an important role in protective immunity to virus infections. They recognize their epitopes as short peptides associated with major histocompatibility complex (MHC) class I molecules after intracellular processing of viral proteins and transport of these peptides to the surface of infected cells. Virus-specific CTL vary in their ability to recognize and kill their target cells and control infection. It has been shown that $CD8^+$-T-cell functional avidity can influence the efficacy of antigen-specific CTL (1, 2, 18, 20, 30, 42, 45, 51, 54). Functional avidity is defined by the amount of peptide required for T-cell activation or effector function (1, 2, 18). It is of special interest that CTL with high functional avidity, which control simian immunodeficiency virus (SIV) infections most effectively, also select for CTL escape mutant viruses (30). Apparently high-functional-avidity CTL exerted the highest selective pressure against SIV CTL epitopes, resulting in the emergence of these escape mutants.

Accumulation of amino acid substitutions in or adjacent to CTL epitopes is an efficient strategy that viruses exploited to escape from CTL immunity and has been demonstrated for viruses such as human immunodeficiency virus (HIV) (12, 22, 23, 27, 32, 34, 35), hepatitis C virus (13, 49), and lymphocytic choriomeningitis virus (28, 33), which are known for their high mutation rates. Influenza A viruses, which cause annual outbreaks of acute respiratory tract infections, also display high mutation rates, allowing the virus to escape from humoral immunity mediated by virus-neutralizing antibodies specific for the membrane glycoproteins (44). Influenza virus-specific CTL contribute to protective immunity against infection, since it was demonstrated that virus secretion after experimental infection of humans inversely correlated with CTL activity in vitro (26). We have identified a number of amino acid substitutions in the viral nucleoprotein (NP) that were associated with escape from human CTL (10, 40, 48). These variable epitopes include the $NP_{380-388}$, $NP_{383-391}$, and $NP_{418-426}$ epitopes, restricted by HLA-B*0801, -B*2705, and -B*3501, respectively. A fourth epitope with an amino acid substitution associated with escape from CTL is derived from the nonstructural protein 1 (NS1), $NS1_{122-130}$, and is restricted by HLA-A*0201 (unpublished observation).

In the human population, the mutations in these epitopes can reach fixation rapidly, which was explained by small intrahost advantages and population dynamics (21). Indeed, it was shown that the loss of a single epitope affected the human influenza virus-specific CTL response in vitro significantly (4). Thus, although the mechanisms may be different, both in chronic infections and during acute infections with a virus that causes epidemics annually in a significant proportion of the population, $CD8^+$ T lymphocytes can drive diversification of viruses (21, 31).

The $NP_{418-426}$ epitope in particular displayed a remarkable degree of variation with amino acid substitutions at four dif-

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TABLE 1. HLA-A and -B genotypes of 18 individuals studied

Group	No. of individuals	Genotypes of HLA locus	
		A*	B*
I	5	0101, 0201	0801, 3501
II	7	0101, 0201	0801, 2705
III	5	0101, 0301	0801, 3501
IV	1	0101, 0201	0801, 3503

ferent positions of the 9-mer. Eight different variants were identified in the influenza sequence database (http://www.flu.lanl.gov), and three of these were associated with escape from virus-specific CTL (10, 11).

We hypothesized that influenza A virus-specific CTL that recognize their target cells with high functional avidity also exert the highest selective pressure on the epitope sequences that are recognized. To test this hypothesis, we determined the functional avidity of polyclonal influenza virus-specific T cell populations directed to nine epitopes.

The low frequency of influenza virus-specific CTL in the blood necessitated the expansion of virus-specific T cells. Since it was found that the hierarchy of peptide-specific CTL responses and the HLA preference measured ex vivo in peripheral blood mononuclear cells (PBMC) correlated with that of the CTL activity measured after in vitro expansion of virus-specific CTL (8, 9), we used expanded CTL for the determination of immunodominance and functional avidity. We found that the functional avidity of influenza virus epitope-specific CTL correlated with the immunodominance of these epitopes. In addition, the hypervariable $NP_{418-426}$ epitope was recognized by CTL with higher functional avidity than other epitopes.

MATERIALS AND METHODS

Cells, virus, and peptides. A total of 18 healthy individuals, divided into four groups, were selected according to serologic homology within the A and B loci of HLA class I molecules (Table 1). Genetic subtyping was performed using a commercial typing system (Genovision, Vienna, Austria). PBMC were isolated from whole blood by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation and cryopreserved at −135°C. All donors were between 35 and 50 years of age and had serum antibodies against one or more influenza A virus strains, measured by hemagglutination inhibition assay, indicative of one or more exposures in the past (8, 9).

Sucrose gradient-purified influenza A virus (H3N2) Resvir-9, a reassortant of influenza virus A/Nanchang/933/95 and A/Puerto Rico/8/34, was used for infection of PBMC. This virus contains the CTL epitopes described in Table 2. Synthetic peptides (immunograde > 85%) representing these epitopes were manufactured, high-performance liquid chromatography purified, and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). Peptides were dissolved in dimethyl sulfoxide (5.0 mg/ml), diluted in RPMI 1640 medium (Cambrex, Heerhugowaard, The Netherlands) to 100 μM, and stored at −20°C until further use.

Of each group of HLA-A- and -B-matched donors, Epstein-Barr virus-transformed B-lymphoblastoid cell lines (BLCL) were established as described previously (41). BLCL were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), 2 mM glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin (R10F; Cambrex, Heerhugowaard, The Netherlands).

In vitro stimulation of PBMC with influenza A virus. Stimulation of PBMC with influenza A virus was performed as described previously (9). Cells were resuspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin (R10F) and infected with Resvir-9 at a multiplicity of infection of 3. After 1 h at 37°C, the cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% pooled human AB serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 20 μM 2-mercaptoethanol (R10H) and added to uninfected PBMC at a ratio of 1:1 in a 25-cm^2 culture flask at a final concentration of 1×10^6 cells/ml. After 2 days, 20 U/ml recombinant human interleukin-2 (Chiron B.V., Amsterdam, The Netherlands) was added, and the cells were incubated for another 7 days at 37°C.

TABLE 2. Influenza A virus CTL epitopes used in this study[a]

Epitope	Antigen[b]	Restriction element	Conserved (reference)
CTELKLSDY	NP_{44-52}	HLA-A1	Yes
VSDGGPNLY	$PB1_{591-599}$	HLA-A1	Yes
GILGFVFTL	$M1_{58-66}$	HLA-A*0201	Yes
AIMDKNIIL	$NS1_{122-130}$	HLA-A*0201	No (unpublished data)
ILRGSVAHK	$NP_{265-273}$	HLA-A3	Yes
ELRSRYWAI	$NP_{380-388}$	HLA-B*0801	No (39)
SRYWAIRTR	$NP_{383-391}$	HLA-B*2705	No (47)
RRSGAAGAAVK	$NP_{174-184}$	HLA-B27	Yes
LPFEKSTVM	$NP_{418-426}$	HLA-B*3501	No (10)

[a] Epitopes are referred to by the antigen and the position of the first amino acid within the protein. Epitopes were considered conserved when they contained no mutations that reached fixation or which resulted in abrogation of recognition by specific CTL. If this was the case, they were considered variable (10, 40, 48).

[b] Subscripts indicate amino acid positions.

Functional avidity of epitope-specific cells. The functional avidity of influenza A virus-specific effector cells was assessed using the enzyme-linked immunospot (ELIspot) avidity assay. This assay allowed us to quantify the number of effector T cells producing gamma interferon (IFN-γ) upon peptide stimulation. Autologous BLCL or HLA-A- and HLA-B-matched BLCL were used as stimulator cells. Thirty thousand cells of BLCL were transferred into 96-well V-bottom plates in 100 μl of R10F and incubated with 50 μl of 10-fold serially diluted peptide solutions at end concentrations ranging from 10^{-11} M to 10^{-5} M. Untreated BLCL were included as controls. Each peptide was diluted and tested in duplicate within a single assay. After a 1-h incubation at 37°C, the cells of the BLCL were washed once with R10F and incubated with 1.5×10^4 influenza A virus-specific effector cells for 1 h at 37°C after centrifugation of the plates for 1 min at $130 \times g$. Finally, the cells were transferred to SilentScreen ELIspot plates (Nalge Nunc International, Rochester, NY) coated overnight with IFN-γ capture antibody (1-DIK; Mabtech, Uppsala, Sweden) in phosphate-buffered saline at 4°C and subsequently blocked with R10H for 2 h at 37°C. After 5 h at 37°C, the cells were removed by vigorous washing, and the assay was further developed. Spots were counted using an AELVIS reader (Sanquin Bloodbank at CLB, Amsterdam, The Netherlands) and plotted as percentages of the maximum response against peptide concentration. These plots were used to determine the peptide concentration giving 50% of maximum response, also referred to as 50% effective concentration (EC_{50}).

^{51}Chromium release assay. The functional avidity of effector cells was also assessed using a classical ^{51}Cr release assay, measuring lysis of target cells. Autologous BLCL were used as target cells. BLCL cells (10^6) were washed once in RPMI medium containing 0.1% bovine serum albumin (R0.1B) and incubated for 1 h at 37°C with 75 μCi $Na_2{}^{51}CrO_4$. Next, the cells were washed twice in R10F and distributed in 96-well V-bottom plates (5,000 cells/well). After addition of the 10-fold serially diluted peptide solutions, the cells were incubated for 1 h at 37°C. The peptide concentrations ranged from 10^{-11} M to 10^{-5} M. A medium control was included to determine the background target cell lysis. Next, the cells were washed once in R10F and used as target cells in a ^{51}Cr release assay as described previously (9). Influenza A virus-specific effector cells were added to the target cells at an effector-to-target cell ratio of 20:1 or 5:1, respectively. After 4 h at 37°C, the culture supernatants were harvested (Skatron instruments, Sterling, Va.) and radioactivity was measured by gamma counting. The percent lysis was calculated with the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Peptide-specific lysis was calculated using the percent lysis of peptide-pulsed target cells minus the percent lysis of unpulsed or control target cells of at least three wells. The EC_{50} was expressed as the concentration of peptide mediating 50% of maximum recognition.

Statistical analysis. The donor in group IV was HLA-B*3503 positive and therefore excluded from any analysis regarding the HLA-B*3501-restricted NP_{418} epitope. The EC_{50} was calculated when the number of spots in the well with the highest concentration of peptide (10^{-5} M) was at least three times

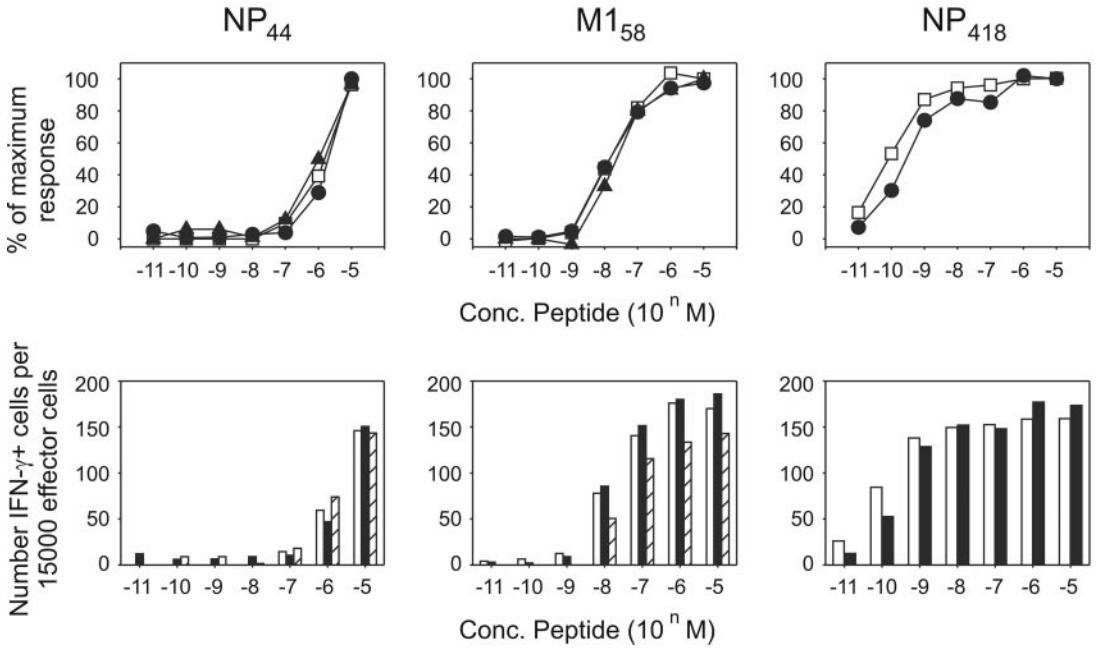

FIG. 1. Use of different BLCL for the measurement of functional avidity. The functional avidity profiles of CTL specific for the HLA-A1-restricted NP_{44} epitope obtained from donor 7, the HLA-A*0201-restricted $M1_{58}$ epitope obtained from donor 7, and the HLA-B*3501-restricted NP_{418} epitope from donor 1 were determined using various BLCL. For each CTL epitope, the autologous and one or two HLA-matched heterologous BLCL were used. (A) Number of epitope-specific IFN-γ^+ cells per 15,000 effector cells calculated as a percentage of the maximum response; (B) number of epitope-specific IFN-γ^+ cells per 15,000 effector cells following stimulation with the different BLCL pulsed with the NP_{44}, $M1_{58}$, or NP_{418} epitope. The use of autologous BLCL is indicated by white squares and white bars; heterologous BLCL are indicated by black circles and black bars or black triangles and hatched bars. Conc., concentration.

higher than the number of spots in the control wells. Statistical significance of the differences in EC_{50} or the number of epitope-specific IFN-γ^+ cells per 15,000 effector cells between variable and conserved epitopes was determined using analysis of variance. A similar statistical analysis in combination with a Bonferroni post hoc analysis was used to analyze differences between the EC_{50}s of individual epitopes. Statistical analysis on the correlation between EC_{50}s and the number of epitope-specific spots per 15,000 effector cells was done with a Pearson correlation test. Prior to statistical analysis, the EC_{50}s were ln transformed. A P value of <0.05 was considered statistically significant.

RESULTS

Validation of the ELIspot functional avidity assay. The peptide concentration required to induce IFN-γ production in 50% of the epitope-specific T cells (EC_{50} or functional avidity) was determined in 18 subjects. To this end, an ELIspot assay was used to measure IFN-γ production in polyclonal T cell populations after stimulation with BLCL pulsed with various concentrations of the peptides.

First, the effect of different BLCL as stimulator cells on the EC_{50} was determined (Fig. 1). One or two different HLA-matched BLCL were tested along with autologous BLCL for their ability to induce a response in NP_{44}-, $M1_{58}$-, and NP_{418}-specific CTL. In donor 7, the EC_{50} of NP_{44}-specific T cells, following stimulation with NP_{44} pulsed autologous BLCL, was 1×10^{-6} M (Fig. 1). Little to no difference in EC_{50} was found for this epitope following stimulation with two other HLA-A1-positive BLCL. The EC_{50}s for the $M1_{58}$ and NP_{418} epitopes were 1.0×10^{-8} M and 3.0×10^{-10} M, respectively, regardless of whether autologous or nonautologous HLA-matched BLCL were used for stimulation. Also the frequency of epitope-specific CTL was similar after stimulation with various HLA-matched BLCL (Fig. 1). Based on these data, it was decided to use a single HLA-matched BLCL for all donors within one group.

To determine the interassay variation, functional avidity of NP_{44}- and $M1_{58}$-specific T cells of donor 7 and the NP_{418}-specific T cells of donor 1 was determined. EC_{50}s were in the same order of magnitude in repeated experiments for the NP_{44}-, $M1_{58}$-, and NP_{418}-specific T cells. We thus concluded that comparison of data obtained in different assays was acceptable (Table 3). The number of epitope-specific IFN-γ^+ cells per 15,000 effector cells was also similar in all three repeated experiments.

The ELIspot functional avidity assay is based solely on the expression of IFN-γ as a result of CTL activation. Since it is

TABLE 3. Reproducibility of the functional avidity ELIspot assay

Epitope	Assay no.	No. of epitope-specific T cells[a]	Functional avidity (M)
NP_{44}	1	172	8×10^{-7}
	2	185	6×10^{-7}
	3	125	7×10^{-7}
$M1_{58}$	1	170	5×10^{-8}
	2	216	1×10^{-8}
	3	180	1×10^{-8}
NP_{418}	1	134	1×10^{-10}
	2	170	9×10^{-10}

[a] The number of IFN-γ^+ peptide-specific T cells per 15,000 effector cells after stimulation with BLCL pulsed with 1.0×10^{-5} M peptide.

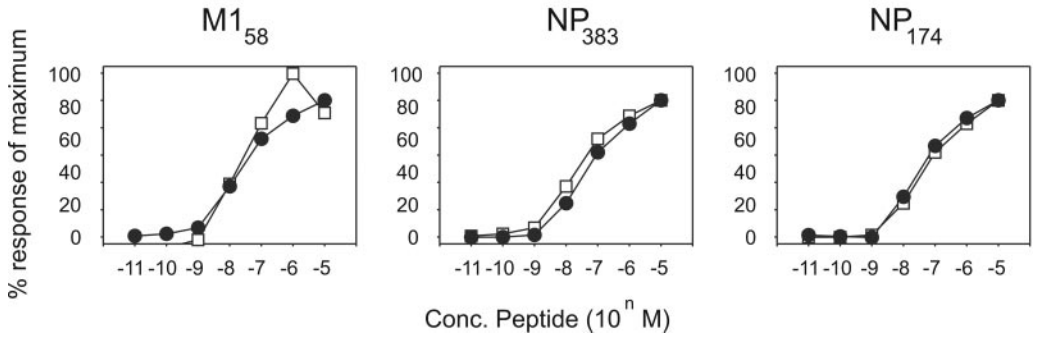

FIG. 2. Comparison of functional avidity measured in ^{51}Cr release assay and ELIspot assay. The functional avidity of CTL specific for the HLA-A*0201-restricted $M1_{58-66}$ epitope ($M1_{58}$) and the two HLA-B*2705-restricted $NP_{383-391}$ (NP_{383}) and $NP_{174-184}$ (NP_{174}) epitopes from donor 7 was determined using a ^{51}Cr release assay and the ELIspot assay. The percentage of specific lysis or number of spots is presented as the percentage of the maximum response. Squares and circles represent the ^{51}Cr release assay and the ELIspot assay, respectively. Conc., concentration.

known that in some cases the thresholds for activation of CTL differ for the production of cytokines and the ability to lyse cells (6), classical ^{51}Cr release assays were performed for comparison. For the three epitopes tested, similar EC_{50}s were obtained from the ^{51}Cr release assay and the ELIspot assay (Fig. 2).

Functional avidity of influenza A virus epitope-specific CTL. The functional avidity of in vitro-expanded virus-specific polyclonal CTL populations obtained from 18 HLA-typed individuals was tested in ELIspot assays using nine peptides representing influenza A virus epitopes (Table 1). For each of the peptides, a minimum of five (NP_{265}) and a maximum of 15 (NP_{380}) different EC_{50}s were obtained. The majority (78%) of the 82 different EC_{50}s obtained were determined twice in independent experiments. In these cases the average was calculated.

The average EC_{50} for these epitopes ranged from 2.7×10^{-10} M (NP_{418}) to 3.9×10^{-7} M (NP_{265}), spanning a 3-log range in epitope concentration (Fig. 3A and Table 4). The average EC_{50} of the NP_{418} epitope was significantly lower that the EC_{50}s of all other epitopes ($P < 0.01$). The EC_{50} of the NP_{383} epitope differed significantly from those of the $PB1_{591}$ and NP_{265} epitopes, and the values for the $M1_{58}$ differed significantly from those found for NP_{265} and $PB1_{591}$. All other comparisons did not yield statistical significant differences.

Of the nine influenza A virus CTL epitopes tested in this study, four (NP_{418}, NP_{380}, NP_{383}, and $NS1_{122}$) displayed amino acid variation associated with escape from CTL. The EC_{50}s of these four variable epitopes were significantly lower than those of the five conserved epitopes ($P < 0.01$) (Fig. 3B).

The homogeneity of functional avidity of polyclonal epitope-specific CTL responses in the different donors varied between the nine influenza A virus epitopes. The difference between the minimum and maximum EC_{50}s for the NP_{174}, NP_{418}, $PB1_{591}$, and NP_{383} epitopes was a 5-, 8-, 10- and 11-fold dilution of the peptide (Table 4). This suggests that these epitope-specific T cells, although from different donors, respond to similar epitope densities. CTL specific for the $M1_{58}$, NP_{380}, or NP_{265} epitopes also reacted to similar epitope densities, although larger differences between minimum and maximum were observed (20-fold dilution of the peptide). The largest difference in EC_{50} was observed for NP_{44}- and $NS1_{122}$-specific CTL (83- and 2,500-fold dilution, respectively).

Based on their HLA-A and HLA-B haplotypes, the study subjects were divided into four groups (Table 1). The EC_{50}s for a single epitope restricted by a shared HLA allele did not differ significantly between the groups (data not shown).

Hierarchy of immunodominance of influenza A virus-specific CTL. The number of spots or IFN-γ^+ cells per 15,000 effector cells obtained after stimulation with 10^{-5} M peptide was used to determine the hierarchy of immunodominance among the different influenza A virus CTL epitopes. Based on single frequencies of epitope-specific IFN-γ^+ cells per 15,000 effector cells, the average number of epitope-specific IFN-γ^+ cells per 15,000 effector cells in all 18 subjects was determined. The HLA-B*2705-restricted NP_{383} epitope had the highest average number of IFN-γ^+ cells (114 IFN-γ^+ cells) per 15,000 effector cells, followed by both the HLA-A*0201-restricted $M1_{58}$ (100 IFN-γ^+ cells) and the other HLA-B*2705-restricted epitope NP_{174} (95 IFN-γ^+ cells) (Table 4). Low frequencies of epitope-specific cells per 15,000 effector cells were obtained for the HLA-A*0201-restricted NS_{122} epitope (29 IFN-γ^+ cells), the HLA-A1-restricted $PB1_{591}$ epitope (35 IFN-γ^+ cells), and the HLA-A3-restricted NP_{265} epitope (45 IFN-γ^+ cells). The remaining three epitopes, namely the HLA-B*0801-restricted NP_{380} epitope (55 IFN-γ^+ cells), the HLA-A1-restricted NP_{44} epitope (65 IFN-γ^+ cells), and the HLA-B*3501-restricted epitope NP_{418} (74 IFN-γ^+ cells), had intermediate numbers of IFN-γ^+ cells per 15,000 effector cells (Table 4). There was no significant difference between the number of IFN-γ^+ cells per 15,000 effector cells of T cells specific for conserved epitopes and that of T cells specific for variable epitopes (Fig. 3C).

Correlation between functional avidity and T-cell hierarchy of immunodominance of influenza A virus epitope-specific CTL. Finally, the correlation between EC_{50} or functional avidity and the frequency of epitope-specific T cells was investigated. A weak correlation ($P = 0.28$, $R^2 = 0.16$) (Fig. 4D) was found when the average values for both conserved and variable epitopes were included in the analysis. A similar analysis was done on the conserved and variable epitopes alone (Fig. 4E

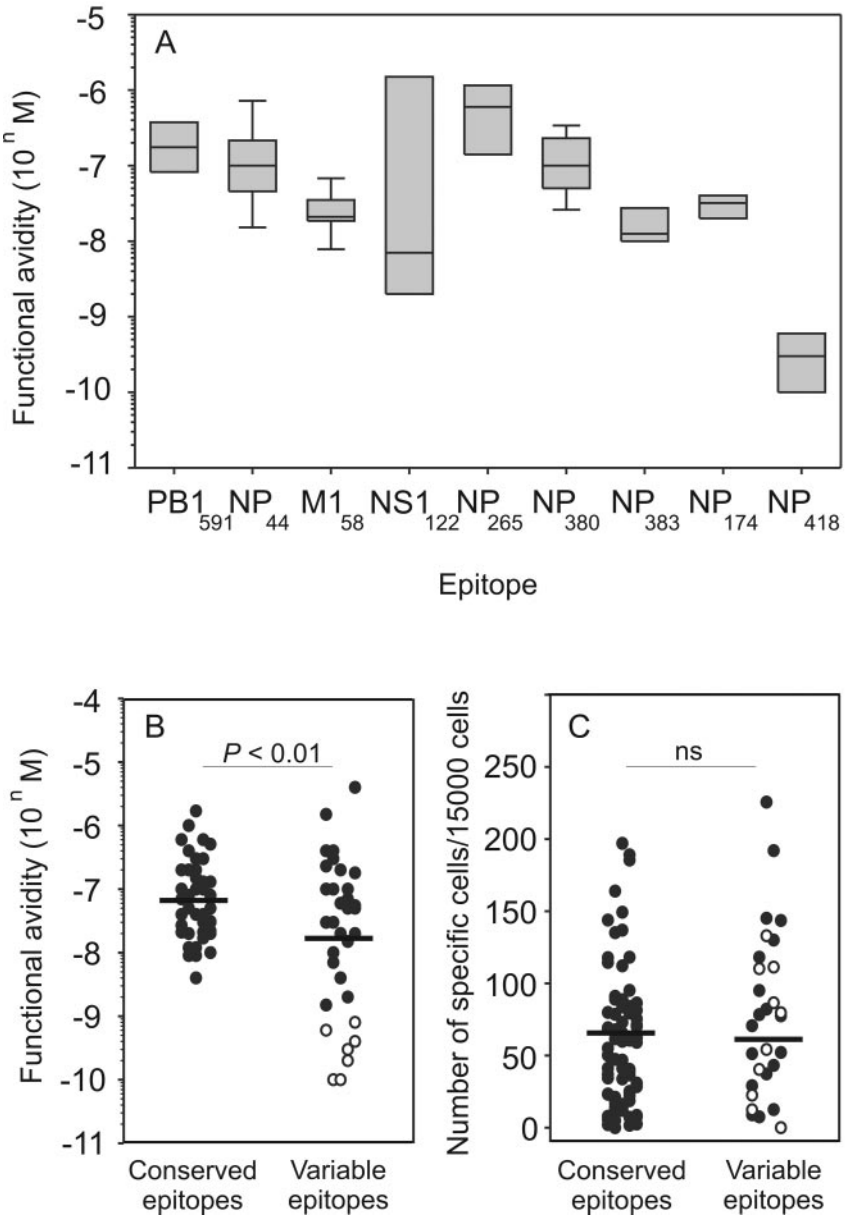

FIG. 3. Functional avidity of influenza A virus epitope-specific CTL. The functional avidity (EC_{50}) of influenza A virus epitope-specific CTL for their respective epitopes was determined for 18 individuals. (A) A box plot was generated from the data. The line in the box represents the median EC_{50}, while the size of the box (gray area) represents the 25th to 75th percentiles of the data. The error bars represent the 10th to 90th percentiles. (B) Functional avidity of in vitro-expanded influenza A virus-specific CTL populations directed to conserved and variable epitopes. (C) Frequency of influenza A virus-specific CTL directed to conserved and variable epitopes in 15,000 in vitro-expanded effector cells. The black line in panels B and C represents the average EC_{50} or number of epitope-specific IFN-γ^+ cells per 15,000 effector cells, respectively. The open circles represent the data obtained for CTL specific for the NP_{418} epitope.

and F). A significant correlation between EC_{50} and the frequency of epitope-specific T cells was observed for the conserved epitopes ($P < 0.05$, $R^2 = 0.84$) (Fig. 4E). For the variable epitopes, no correlation between EC_{50} and the frequency of epitope-specific T cells was demonstrated ($P = 0.71$, $R^2 = 0.08$) (Fig. 4F). Analysis based on the scatter plots shown in Fig. 4A to C, representing all individual data points obtained from all study subjects and for all peptides tested, essentially gave the same result.

DISCUSSION

In the present study it was demonstrated that NP_{418}-specific polyclonal CTL populations recognized this hypervariable epitope with high functional avidity.

Since it was shown that CTL, which recognize their epitopes with high functional avidity are superior in eliminating virus-infected cells or tumor cells (1, 2, 18, 20, 30, 42, 45, 51, 54), we hypothesized that CTL with high functional avidity could exert

TABLE 4. Average frequency and functional avidity of influenza A virus epitope-specific CTL in a group of 18 individuals

Epitope	Frequency		Functional avidity	
	Mean ± SEM (range)[a]	n[b]	Mean (M) ± SEM (range)	n[b]
NP_{418}	74 ± 18.2 (0–175)	10	$2.7 \times 10^{-10} \pm 1.0 \times 10^{-10}$ (1.0×10^{-10}–8.0×10^{-10})	7
NP_{383}	114 ± 30.9 (7–225)	7	$1.6 \times 10^{-8} \pm 6.4 \times 10^{-9}$ (1.0×10^{-8}–5.0×10^{-8})	6
$M1_{58}$	100 ± 11.9 (46–173)	13	$2.3 \times 10^{-8} \pm 5.4 \times 10^{-9}$ (8.0×10^{-8}–9.0×10^{-9})	13
NP_{174}	95 ± 19.4 (28–185)	7	$2.9 \times 10^{-8} \pm 1.1 \times 10^{-8}$ (1.0×10^{-7}–6.0×10^{-9})	7
$NS1_{122}$	29 ± 9.8 (0–106)	13	$3.4 \times 10^{-8} \pm 5.7 \times 10^{-7}$ (4.0×10^{-6}–1.5×10^{-9})	7
NP_{44}	65 ± 11.6 (2–196)	18	$9.4 \times 10^{-8} \pm 7.2 \times 10^{-8}$ (1.0×10^{-6}–1.2×10^{-8})	13
NP_{380}	55 ± 9.8 (8–162)	18	$9.9 \times 10^{-8} \pm 3.0 \times 10^{-8}$ (4.0×10^{-7}–2.0×10^{-8})	15
$PB1_{591}$	35 ± 8.4 (2–136)	18	$1.7 \times 10^{-7} \pm 5.8 \times 10^{-8}$ (5.1×10^{-7}–5.0×10^{-8})	8
NP_{265}	45 ± 7.1 (25–62)	5	$3.9 \times 10^{-7} \pm 2.9 \times 10^{-7}$ (1.7×10^{-6}–8.0×10^{-8})	5

[a] Average frequency of epitope-specific CTL per 15,000 effector cells.
[b] n, number of individuals used to determine the frequency or functional avidity of each influenza A virus CTL epitope.

higher selective pressure on influenza viruses and therefore drive diversification of CTL epitopes more efficiently than low-functional-avidity CTL, allowing the virus to escape from recognition by specific CTL.

First, we established an assay with which we could determine functional avidity of CTL specific for nine different epitopes in a high-throughput fashion. We wished to use only a limited number of HLA-matched BLCL as stimulator cells for the induction of IFN-γ in the effector cell populations, and therefore, we demonstrated that similar EC_{50}s were obtained regardless of the BLCL that was used for stimulation. In addition, the values obtained in the IFN-γ ELIspot assay were similar to those obtained in another functional test, the classical ^{51}Cr release assay. These results are in contrast with previous observations which indicated that the induction of cytolytic activity and IFN-γ production in cytomegalovirus- and HIV-specific CTL do not have the same peptide concentration thresholds (6). These differences may be explained by

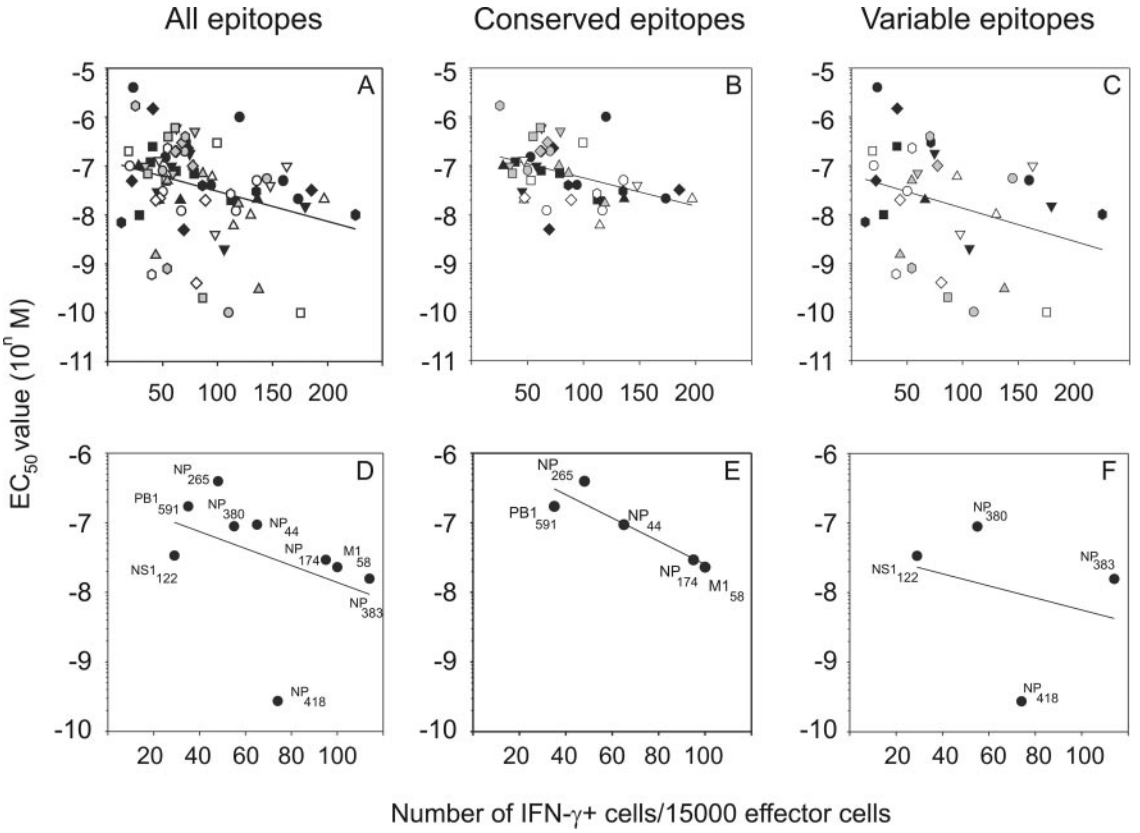

FIG. 4. Regression analysis of functional avidity and frequency of influenza A virus epitope-specific CTL. Regression analysis between functional avidity (EC_{50} in M) and the frequency of epitope-specific CTL (number of epitope-specific IFN-γ^+ cells per 15,000 effector cells) performed on all available data points (each symbol represents a peptide) obtained from all study subjects tested for all nine epitopes (A), the conserved epitopes (B), or the variable epitopes (C) and on the average values for all nine epitopes (D), the conserved epitopes (E), and the variable epitopes (F).

the nature of infection (chronic versus acute), the viruses and/or the epitopes that were studied, or the source and nature of the T cells that were used for the analysis (ex vivo T cells versus in vitro-expanded T cells) (7). We wanted to use polyclonal effector cell populations, which were obtained after stimulation with virus-infected cells, since this would better reflect the in vivo situation than, for example, the use of single T-cell clones specific for the nine peptides that were tested. Indeed, individual CTL clones that recognize a particular epitope could do so with different functional avidities (50). However, we have tested the EC_{50}s for some T-cell clone-peptide combinations and these were in the same order of magnitude as those observed with the polyclonal T cell populations (data not shown).

The data obtained with PBMC in vitro may also have implications for peripheral T-cell memory in the airways, since it has been shown that memory T-cell populations in the lung are recruited from the circulation (19).

Previously, it has been shown that functional avidity can mature during the course of acute infection (43). Since, in our study, PBMC were collected outside the epidemic period without any influenza activity, it is unlikely that the study subjects experienced acute infections. Most likely, virus-specific memory CTL were studied that had reached their maximum functional avidity.

The range of EC_{50}s varied widely with the CTL specific for the NP_{265} epitope on the high end and those specific for the NP_{418} epitope on the low end of the spectrum. Seven of the nine epitope-specific CTL populations exhibited a narrow range of functional avidity. This may reflect the oligoclonal nature of these CTL responses, as was demonstrated for the $M1_{58}$-specific CTL response, which is dominated by T cells carrying the $V\beta17^+$ T-cell receptor (25, 29).

We feel that it is conceivable that viruses have an advantage if they can escape from CTL with high functional avidity that require up to 1,000 times less MHC class I peptide complexes for their activation than some of the other CTL. A time window is created in which (mutant) virus replication is unaffected by these efficient CTL, allowing more progeny virus to be produced until CTL with lower functional avidity ultimately eliminate the infected cell. In this scenario, more virus is produced during a longer period of time.

Others have demonstrated with a number of different CTL clones specific for various HIV proteins that their antiviral efficiency is not dependent on their functional avidity per se but on that of the protein that is recognized. In particular, CTL specific for the Nef protein, which is expressed early in the virus replication cycle, were more efficient in inhibiting virus replication (50). Indeed, the time point after infection at which HIV-infected cells became sensitive to CTL-mediated killing had a profound effect on virus shedding in vitro and in vivo (46, 47). Although in the latter studies the functional avidity was not studied, they clearly indicated that the kinetics of CTL recognition was determined by the kinetics of protein expression in the infected cells. Of course, the kinetics of lentivirus replication do not apply to influenza virus replication, where all proteins are expressed within 4 h postinfection. Therefore, CTL with high functional avidity may be more important in the control of influenza virus than of HIV.

Thus, escape from high functional avidity CTL seems a plausible scenario from which the virus benefits the most. This reasoning is in agreement with findings in the SIV macaque model (30, 31). It was found that CTL with high functional avidity eliminated virus-infected cells most efficiently and exerted strong intrahost selection. As a result, mutations were observed in the epitopes recognized by high-functional-avidity CTL, allowing the virus to escape recognition by these CTL. Thus, this chronic infection model showed that there was a strong correlation between T-cell functional avidity and variability in CTL epitopes.

The superior effector function of T cells with high functional avidity have been documented previously, and it most likely is related to multiple mechanisms for the elimination of virus-infected cells (18), which in turn may contribute to the emergence of CTL escape mutants, as was shown in mice transgenic for the T-cell receptor specific for the influenza A virus $NP_{366-374}$ peptide (36).

Following respiratory infection with simian virus 5 or vaccinia virus, it was found that the CTL response early after infection is of high functional avidity compared to CTL that arise later after infection (24). In contrast, CTL specific for the Epstein-Barr virus early antigens recognized their epitopes with lower functional avidity and were more immunodominant and more efficient in eliminating infected cells than those specific for late antigens. Probably the efficiency of antigen processing during the lytic stage of the herpes virus infection cycle had affected recognition by CTL specific for the L antigens (37).

We now provide evidence that influenza viruses that cause acute respiratory tract infections in a significant portion of the human population are also subject to diversification in CTL epitopes driven by CTL with high functional avidity when immune pressure in the population is sufficiently high. In a population that has been exposed multiple times to the virus and in which a strong CTL− herd immunity has been established, a difference of a couple of hours before infected cells become sensitive to CTL killing could result in a marked advantage to the virus in selected individuals. In a theoretical model, prolonged viral shedding in a small portion of the human population with the corresponding HLA haplotype provided sufficient advantage for rapid fixation of the CTL escape mutants at the population level (21). Other factors may also contribute to the variability in CTL epitopes. Of special interest is the fact that some of the variable epitopes overlap and are presented by different HLA molecules, like the NP_{380} and NP_{383} epitopes. The NP_{418} epitope was also found to bind to HLA-B7 in addition to HLA-B*3501 (16). Despite the evidence for selective pressure in 9-mer amino acid sequences constituting influenza virus CTL epitopes (5) and for a correlation between CTL functional avidity and variability in CTL epitopes (this study), there are exceptions. For example, the HLA-A*0201-restricted $M1_{58-66}$ epitope is immunodominant, elicits CTL with high functional avidity in a large portion of the human population, and yet, is highly conserved. A mutational analysis of this epitope indicated that this epitope is under functional constraints, which may limit escape from CTL. It is of interest that alanine replacements were tolerated to certain extents at each residue of the 9-mer except for the second, which constitutes the anchor residue of the epitope. More conservative substitutions at this position had less dramatic effects on viral fitness but did not prevent recognition by $M1_{58-66}$-specific CTL

(5). The amino acid substitution at the anchor residue of the HLA-B*2705-restricted $NP_{383-391}$ epitope appeared detrimental to viral fitness and was only tolerated in the presence of functionally compensating comutations (38, 39). These findings indicate that the virus cannot simply evade host immunity at the cost of viral fitness. In this respect, it is of interest that the otherwise hypervariable NP_{418} epitope retained its anchor residues, which could not be replaced without the loss of viral fitness (5).

The functional avidity of CTL specific for conserved influenza virus epitopes also correlated with the magnitude of the CTL responses in vitro. This finding is in concordance with previous studies that showed a correlation between the functional avidity and the immunodominance of the CTL response specific for minor histocompatibility antigens in a mouse model (53).

It is possible that the study subjects had been exposed less frequently to mutant CTL epitopes than conserved epitopes, which may have affected the magnitude of the CTL responses in vitro. This might explain the loss of correlation between functional avidity and immunodominance when the variable epitopes are included in the comparison (Fig. 4).

Studies on influenza A virus-specific CTL responses in mice also identified a role for functional avidity in establishing immunodominance hierarchies among various epitope-specific T cells (3, 14). However, it is known that the hierarchy of primary and secondary CTL response can differ and other factors, like epitope abundance, differential antigen presentation, or limitations in the T-cell response must be involved (3, 14, 15, 17).

It is likely that immunodominant epitopes also bind to their corresponding MHC class I molecules with high affinity, which in turn could result in preferential peptide loading in the endoplasmic reticulum and sustained presentation to specific CTL. Mathematical models also suggested that small differences in the initial time of activation have a large impact on the magnitude of the response (52).

We hypothesize that in the battle between influenza virus and its host, the virus evades recognition by CTL with high functional avidity by accumulating amino acid substitutions in CTL epitopes, allowing the virus to replicate to higher titers and for prolonged periods of time in selected individuals. The diversification in epitopes recognized by CTL with high functional avidity might be of importance for the successful persistence of influenza A viruses in the human population.

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REFERENCES

1. **Alexander-Miller, M. A.** 2005. High-avidity CD8+ T cells: optimal soldiers in the war against viruses and tumors. Immunol. Res. **31:**13–24.
2. **Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky.** 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci. USA **93:**4102–4107.
3. **Belz, G. T., W. Xie, J. D. Altman, and P. C. Doherty.** 2000. A previously unrecognized H-$2D^b$-restricted peptide prominent in the primary influenza A virus-specific $CD8^+$ T-cell response is much less apparent following secondary challenge. J. Virol. **74:**3486–3493.
4. **Berkhoff, E. G., A. C. Boon, N. J. Nieuwkoop, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2004. A mutation in the HLA-B*2705-restricted $NP_{383-391}$ epitope affects the human influenza A virus-specific cytotoxic T-lymphocyte response in vitro. J. Virol. **78:**5216–5222.
5. **Berkhoff, E. G., E. de Wit, M. M. Geelhoed-Mieras, A. C. Boon, J. Symons, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2005. Functional constraints of influenza a virus epitopes limit escape from cytotoxic T lymphocytes. J. Virol. **79:**11239–11246.
6. **Betts, M. R., D. A. Price, J. M. Brenchley, K. Lore, F. J. Guenaga, A. Smed-Sorensen, D. R. Ambrozak, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek, and R. A. Koup.** 2004. The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. J. Immunol. **172:**6407–6417.
7. **Boon, A. C., G. de Mutsert, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2005. Functional profile of human influenza virus-specific cytotoxic T lymphocyte activity is influenced by interleukin-2 concentration and epitope specificity. Clin. Exp. Immunol. **142:**45–52.
8. **Boon, A. C., G. De Mutsert, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2004. Preferential HLA usage in the influenza virus-specific CTL response. J. Immunol. **172:**4435–4443.
9. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype. J. Virol. **76:**582–590.
10. **Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. J. Virol. **76:**2567–2572.
11. **Boon, A. C., G. de Mutsert, D. van Baarle, D. J. Smith, A. S. Lapedes, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2004. Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes. J. Immunol. **172:**2453–2460.
12. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205–211.
13. **Chang, K. M., B. Rehermann, J. G. McHutchison, C. Pasquinelli, S. Southwood, A. Sette, and F. V. Chisari.** 1997. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J. Clin. Investig. **100:**2376–2385.
14. **Chen, W., L. C. Anton, J. R. Bennink, and J. W. Yewdell.** 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity **12:**83–93.
15. **Chen, W., K. Pang, K. A. Masterman, G. Kennedy, S. Basta, N. Dimopoulos, F. Hornung, M. Smyth, J. R. Bennink, and J. W. Yewdell.** 2004. Reversal in the immunodominance hierarchy in secondary CD8+ T cell responses to influenza A virus: roles for cross-presentation and lysis-independent immunodomination. J. Immunol. **173:**5021–5027.
16. **Cheuk, E., C. D'Souza, N. Hu, Y. Liu, H. Lang, and J. W. Chamberlain.** 2002. Human MHC class I transgenic mice deficient for H2 class I expression facilitate identification and characterization of new HLA class I-restricted viral T cell epitopes. J. Immunol. **169:**5571–5580.
17. **Crowe, S. R., S. J. Turner, S. C. Miller, A. D. Roberts, R. A. Rappolo, P. C. Doherty, K. H. Ely, and D. L. Woodland.** 2003. Differential antigen presentation regulates the changing patterns of CD8+ T cell immunodominance in primary and secondary influenza virus infections. J. Exp. Med. **198:**399–410.
18. **Derby, M., M. Alexander-Miller, R. Tse, and J. Berzofsky.** 2001. High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. J. Immunol. **166:**1690–1697.
19. **Ely, K. H., T. Cookenham, A. D. Roberts, and D. L. Woodland.** 2006. Memory T cell populations in the lung airways are maintained by continual recruitment. J. Immunol. **176:**537–543.
20. **Gallimore, A., T. Dumrese, H. Hengartner, R. M. Zinkernagel, and H. G. Rammensee.** 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J Exp. Med. **187:**1647–1657.
21. **Gog, J. R., G. F. Rimmelzwaan, A. D. Osterhaus, and B. T. Grenfell.** 2003. Population dynamics of rapid fixation in cytotoxic T lymphocyte escape mutants of influenza A. Proc. Natl. Acad. Sci. USA **100:**11143–11147.
22. **Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker.** 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature **412:**334–338.
23. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212–217.
24. **Gray, P. M., G. D. Parks, and M. A. Alexander-Miller.** 2003. High avidity

CD8+ T cells are the initial population elicited following viral infection of the respiratory tract. J. Immunol. **170:**174–181.
25. **Lehner, P. J., E. C. Wang, P. A. Moss, S. Williams, K. Platt, S. M. Friedman, J. I. Bell, and L. K. Borysiewicz.** 1995. Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment. J. Exp. Med. **181:**79–91.
26. **McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare.** 1983. Cytotoxic T-cell immunity to influenza. N. Engl. J. Med. **309:**13–17.
27. **McMichael, A. J., and R. E. Phillips.** 1997. Escape of human immunodeficiency virus from immune control. Annu. Rev. Immunol. **15:**271–296.
28. **Moskophidis, D., and R. M. Zinkernagel.** 1995. Immunobiology of cytotoxic T-cell escape mutants of lymphocytic choriomeningitis virus. J. Virol. **69:** 2187–2193.
29. **Moss, P. A., R. J. Moots, W. M. Rosenberg, S. J. Rowland-Jones, H. C. Bodmer, A. J. McMichael, and J. I. Bell.** 1991. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. Proc. Natl. Acad. Sci. USA **88:** 8987–8990.
30. **O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat. Med. **8:**493–499.
31. **O'Connor, D. H., A. B. McDermott, K. C. Krebs, E. J. Dodds, J. E. Miller, E. J. Gonzalez, T. J. Jacoby, L. Yant, H. Piontkivska, R. Pantophlet, D. R. Burton, W. M. Rehrauer, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2004. A dominant role for $CD8^+$-T-lymphocyte selection in simian immunodeficiency virus sequence variation. J. Virol. **78:**14012–14022.
32. **Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, et al.** 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature **354:**453–459.
33. **Pircher, H., D. Moskophidis, U. Rohrer, K. Burki, H. Hengartner, and R. M. Zinkernagel.** 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. Nature **346:**629–633.
34. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc. Natl. Acad. Sci. USA **94:**1890–1895.
35. **Price, D. A., U. C. Meier, P. Klenerman, M. A. Purbhoo, R. E. Phillips, and A. K. Sewell.** 1998. The influence of antigenic variation on cytotoxic T lymphocyte responses in HIV-1 infection. J. Mol. Med. **76:**699–708.
36. **Price, G. E., L. Huang, R. Ou, M. Zhang, and D. Moskophidis.** 2005. Perforin and Fas cytolytic pathways coordinately shape the selection and diversity of $CD8^+$-T-cell escape variants of influenza virus. J. Virol. **79:**8545–8559.
37. **Pudney, V. A., A. M. Leese, A. B. Rickinson, and A. D. Hislop.** 2005. CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. J Exp. Med. **201:**349–360.
38. **Rimmelzwaan, G. F., E. G. Berkhoff, N. J. Nieuwkoop, R. A. Fouchier, and A. D. Osterhaus.** 2004. Functional compensation of a detrimental amino acid substitution in a cytotoxic-T-lymphocyte epitope of influenza a viruses by comutations. J. Virol. **78:**8946–8949.
39. **Rimmelzwaan, G. F., E. G. Berkhoff, N. J. Nieuwkoop, D. J. Smith, R. A. Fouchier, and A. D. Osterhaus.** 2005. Full restoration of viral fitness by multiple compensatory co-mutations in the nucleoprotein of influenza A virus cytotoxic T-lymphocyte escape mutants. J. Gen. Virol. **86:**1801–1805.
40. **Rimmelzwaan, G. F., A. C. Boon, J. T. Voeten, E. G. Berkhoff, R. A. Fouchier, and A. D. Osterhaus.** 2004. Sequence variation in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. Virus Res. **103:**97–100.
41. **Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus.** 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine **19:**1180–1187.
42. **Sedlik, C., G. Dadaglio, M. F. Saron, E. Deriaud, M. Rojas, S. I. Casal, and C. Leclerc.** 2000. In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity. J. Virol. **74:**5769–5775.
43. **Slifka, M. K., and J. L. Whitton.** 2001. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. Nat. Immunol. **2:**711–717.
44. **Smith, D. J., A. S. Lapedes, J. C. de Jong, T. M. Bestebroer, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2004. Mapping the antigenic and genetic evolution of influenza virus. Science **305:**371–376.
45. **Speiser, D. E., D. Kyburz, U. Stubi, H. Hengartner, and R. M. Zinkernagel.** 1992. Discrepancy between in vitro measurable and in vivo virus neutralizing cytotoxic T cell reactivities. Low T cell receptor specificity and avidity sufficient for in vitro proliferation or cytotoxicity to peptide-coated target cells but not for in vivo protection. J. Immunol. **149:**972–980.
46. **Stittelaar, K. J., R. A. Gruters, M. Schutten, C. A. van Baalen, G. van Amerongen, M. Cranage, P. Liljestrom, G. Sutter, and A. D. Osterhaus.** 2002. Comparison of the efficacy of early versus late viral proteins in vaccination against SIV. Vaccine **20:**2921–2927.
47. **van Baalen, C. A., C. Guillon, M. van Baalen, E. J. Verschuren, P. H. Boers, A. D. Osterhaus, and R. A. Gruters.** 2002. Impact of antigen expression kinetics on the effectiveness of HIV-specific cytotoxic T lymphocytes. Eur. J. Immunol. **32:**2644–2652.
48. **Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan.** 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. J. Virol. **74:**6800–6807.
49. **Weiner, A., A. L. Erickson, J. Kansopon, K. Crawford, E. Muchmore, A. L. Hughes, M. Houghton, and C. M. Walker.** 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc. Natl. Acad. Sci. USA **92:**2755–2759.
50. **Yang, O. O., P. T. Sarkis, A. Trocha, S. A. Kalams, R. P. Johnson, and B. D. Walker.** 2003. Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL. J. Immunol. **171:**3718–3724.
51. **Yee, C., P. A. Savage, P. P. Lee, M. M. Davis, and P. D. Greenberg.** 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. J. Immunol. **162:**2227–2234.
52. **Yewdell, J. W., and M. Del Val.** 2004. Immunodominance in TCD8+ responses to viruses: cell biology, cellular immunology, and mathematical models. Immunity **21:**149–153.
53. **Yoshimura, Y., R. Yadav, G. J. Christianson, W. U. Ajayi, D. C. Roopenian, and S. Joyce.** 2004. Duration of alloantigen presentation and avidity of T cell antigen recognition correlate with immunodominance of CTL response to minor histocompatibility antigens. J. Immunol. **172:**6666–6674.
54. **Zeh, H. J., III, D. Perry-Lalley, M. E. Dudley, S. A. Rosenberg, and J. C. Yang.** 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. J. Immunol. **162:**989–994.